Journal of Natural Products
Vol. 53, No. 6, pp. 1479-1484, Nov-Dec 1990

NEW PROAZULENE GUAIANOLIDES FROM *THAPSIA VILLOSA*

ULLA WAGNER SMITT, CLAUS CORNETT, ANNETTE ANDERSEN, S. BRØGGER CHRISTENSEN,*

PharmaBiotec, Department of Pharmacognosy and Organic Chemistry, Royal Danish School of Pharmacy, Universitetsparken 2, DK-2100 Copenhagen Ø, Denmark

and PINAROSA AVATO

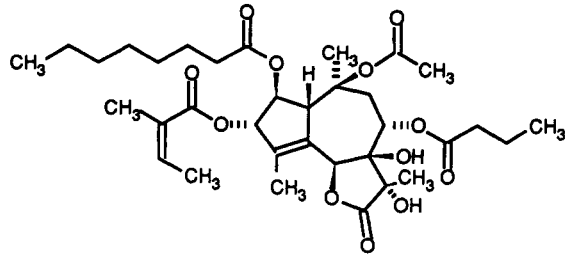
Dipartimento Farmaco-Chimico, Facoltà di Farmacia, Università degli Studi di Bari, I-70126 Bari, Italy

ABSTRACT.—Four new guaianolides **2–5** possessing the terpenoid skeleton of archangelolide [**6**] were isolated from a specimen of *Thapsia villosa* [chromosome number 2n = 66 (= 6x)]. Attempts to hydrolyze the natural products **2–6** yielded azulenes, mainly 7-acetyl-1,4-dimethylazulene [**9**].

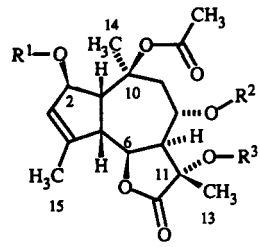
A number of potent skin-irritating histamine secretagogues (e.g., thapsigargin [**1**]) have been isolated from different species of *Thapsia* (1,2). All of the bioactive constituents, which are named thapsigargins, are esters of the hexaoxygenated guaianolide alcohol skeleton of **1** or the pentaoxygenated guaianolide skeleton of trilobolide (1,2). According to *Flora Europaea* (3), the genus *Thapsia* (Umbelliferae) only includes three species: *Thapsia garganica* L., *Thapsia maxima* Mill., and *Thapsia villosa* L. Phytochemical studies, however, have disclosed a pronounced heterogeneity between the three species as well as within them (2, 4–8). These observations have prompted us to undertake a chemotaxonomic investigation. A taxonomic revision of the genus based on a correlation of the pattern of secondary metabolites with morphological characters and chromosome number is approaching.

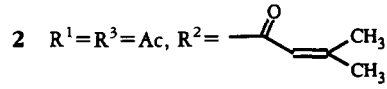
Within *T. villosa*, thapsigargins have only been isolated from roots of plants with chromosome numbers 2n = 44 (= 4x) and 2n = 66 (= 6x), whereas other types of sesquiterpenes have been isolated from specimens with 2n = 22 (= 2x). This paper reports that four new guaianolides **2–5** have been isolated from some specimens of *T. villosa* with 2n = 66 (= 6x). In addition, a relatively low amount of a complex mixture of thapsigargins was detected. Some of the thapsigargins cochromatographed with thapsivillosins A, B, H, and K and thapsitranstagin (1) as evidenced by hplc. Furthermore we want to report that **2–5**, in contrast to the thapsigargins, easily are converted into 7-acetyl-1,4-dimethylazulene [**9**]. None of the new compounds was able to irritate mouse ears nor to induce histamine release.

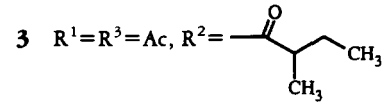
Tlc investigations of EtOH extracts of *T. villosa* [2n = 66 (= 6x)] collected at different places demonstrated the presence of four unknown metabolites. The compounds were purified from roots by cc and hplc. Ir spectroscopy revealed the presence of a γ-lactone ring and of a hydroxy group in the two more polar compounds **4** and **5**. Except for

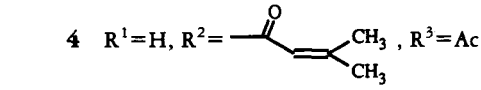


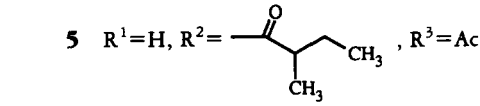
1

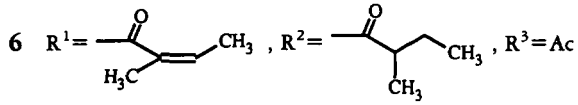


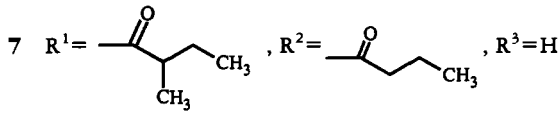












the signals originating in the acyl groups, the ^{1}H-nmr spectra (Table 1) and the ^{13}C-nmr spectra (Table 2) of the two less polar compounds **2** and **3** matched with those of archangelolide [**6**], indicating that **2**, **3**, and **6** are esters of the same tetraoxygenated sesquiterpene lactone (9–11). The structures of the acyl groups in compounds **2** and **3** were deduced from the nmr and eims spectra. Attempts to prove that all the three compounds, **2**, **3**, and **6**, could be converted into the same alcohol by acidic or basic methanolysis failed. In all cases a heavily blue reaction mixture was formed. Pyrolysis of the slovanolide **7** yields 1,4-dimethylazulene [**8**] (12). Thus, it was tempting to speculate that **2**, **3**, and **6** also were transformed into this dye. A gc-ms analysis of the reaction mixture did prove that **8** was formed but only in trace amounts. The major reaction product had a longer retention time and a mol wt 42 units larger than that of **8**. The fact that α-hydroxy carboxylic acids can decarbonylate yielding an oxo compound (13) and the presence of a large peak at *m/z* 43 in the ms suggested that the major blue reaction product formed might be 7-acetyl-1,4-dimethylazulene [**9**]. This hypothesis was substantiated when the reaction product was isolated in a pure state and the ^{1}H-nmr spectrum was found to match the described spectrum of **9** (14). The easy conversion of **2**, **3**, and **6** into **8** and **9** is an additional example of the relatively few known transformations of guaianolides into azulenes, reactions which are important for the understanding of the composition of essential oils (12, 15, 16).

TABLE 1. ^{1}H-nmr Spectra of Compounds **2–6** ($CDCl_3$/TMS).[a]

Proton	Compound	
	2, **3**, and **6**	**4** and **5**
H-1	3.37 dd (3, 9)	2.99 dd (4, 9)
H-2	5.78 broad signal	4.77 broad signal
H-3	5.62 dq (2, 1)	5.64 dq (2, 1)
H-5	3.11 broad t (9, 12)	3.04 broad t (9, 12)
H-6	4.81 dd (12, 10)	4.75 dd (12, 10)
H-7	3.61 dd (10, 11)	3.59 dd (10, 11)
H-8	5.71 dt (11, 3)	5.56 dt (11, 3)
H-9a	2.59 dd (3, 15)	2.66 dd (3, 15)
H-9b	2.06 dd (11, 15)	2.11 dd (11, 15)
H-13	1.62 s	1.58 s
H-14	1.31 s	1.55 s
H-15	1.93 d (1)	1.93 d (1)

[a]Data are δ (ppm), multiplicity, and *J* (in parentheses) in Hz. The signals originating in the acyl groups are found at: acetyl 2.0-2.1 s; 2-methylbutanoyl 2.34 hex (8), 1.72 m, 1.45 m, 1.18 d (8), 0.92 t (8); senecioyl 5.59 sep (1), 2.20 d (1), 192 d (1); angeloyl 1.86 br, 6.05 qq, and 1.95 dq.

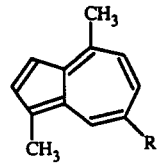
The presence of a hydroxy group in **4** and the high field resonance of the proton assignable to H-2 are explained by the assumption that the only difference between **2** and **4** is a free hydroxy group at C-2 in **4**, whereas this hydroxy group is esterified with HOAc in **2**. Acetylation of **4** to give **2** proved this hypothesis. The senecioyl group of **4** was located by a COLOC experiment, which visualized a coupling between the carbonyl carbon of the senecioyl group and H-8. The location of the senecioyl group in **4** also proved the location of the acyl groups of **2** as depicted.

TABLE 2. ^{13}C-nmr Data for Compounds **2**, **3**, **4**, **5**, and **6** ($CDCl_3$/TMS).[a]

Carbon	Compound				
	2	**3**	**4**	**5**	**6**
C-1	51.9	52.4	57.5	58.4	52.7
C-2	79.9	79.4	77.3	77.1	77.1
C-3	126.7	126.8	129.9	130.3	127.2
C-4	149.5	148.5	146.5	147.1	148.9
C-5	50.2[b]	50.2[b]	49.6[b]	50.1[b]	50.1[b]
C-6	76.3	76.4	76.5	76.6	76.8
C-7	48.4[b]	48.3[b]	47.5[b]	47.9[b]	48.1[b]
C-8	64.7	65.5	64.0	65.1	65.4
C-9	44.9	44.5	43.6	43.8	44.2
C-10	80.7[c]	80.7[c]	81.7[c]	81.9[c]	90.9[c]
C-11	78.0[c]	78.2[c]	77.7[c]	78.1[c]	79.0[c]
C-12	175.0	174.8	173.2	173.5	173.0
C-13	26.6[d]	26.3[d]	26.9[d]	26.3[d]	26.3[d]
C-14	21.2[d]	22.9[d]	21.9[d]	22.3[d]	22.3[d]
C-15	17.4	17.8	17.3	17.9	17.7

[a]Data are δ (ppm). The signals originating in the acyl groups are found at: acteyl 170.3, 169.9, and 169.6, 21.2, 20.4, and 20.2; 2-methylbutanoyl 174.8, 41.3, 26.3, 16.6, 11.5; senecioyl 164.3, 157.7, 115.3, 26.9, 20.3; angeloyl 167.4 s, 126.6 s, 138.0 d, 15.7 q, and 20.4 q.

[b,c,d]These assignments may be interchanged.



8 R=H
9 R=Ac

Analogously, a comparison of the nmr spectra of **3** and **5** leads to the conclusion that these compounds are identical except for the presence of a free hydroxy group at O-2 in **5**, whereas this hydroxy group is acetylated in **3**. Again an acetylation experiment proved this hypothesis. Surprisingly, no coupling between any of the carbonyl carbons and H-8 could be visualized in a COLOC spectrum of **5**. Instead, the suggested location of the acyl groups in compounds **3** and **5** is based on their eims. The major fragmentation in **2** as well as in **6** consists of (a) an elimination of one HOAc $[M-60]^+$, (b) a loss of the acyl group at O-2 as a ketene, (c) an elimination of the acid esterified at O-8, and (d) an elimination of HOAc, yielding eventually a major peak with an *m/z*-value of 244. The assumption that the same fragmentation is dominating in **3** localizes an acetyl group at O-2 and the α-methylbutanoyl group at O-8. Consequently **5** must have the structure depicted.

The structural similarities between **2**, **3**, **4**, **5**, and **1** motivated us to test if the new natural products are skin irritants and histamine secretagogues. None of the new sesquiterpene lactones induced irritation of mouse ear in a dose of 10 μg, and none was able to provoke histamine release from peritoneal rat mast cells in concentrations up to 10 μM. These results confirm the previously advanced structure-activity relationships, which hypothesize that the hydroxy groups on the γ-lactone ring of **1** are essential for the bioactivity (1). The presence of the slovanolides **2–5** in specimens of *T. villosa* seems to be correlated with a significantly decreased amount of thapsigargins. This finding might indicate a common precursor for both types of guaianolides and supports a previously suggested biosynthesis of the unique dihydroxy-γ-lactone of the thapsigargins (17).

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—Cc was performed over acid-washed Si gel, type 60 (Merck), added 10% of H_2O. Tlc was carried out on Merck aluminium-backed tlc sheets (Si gel 60 F_{254}). The tlc systems employed were hexane-EtOAc (14:5), CH_2Cl_2-EtOAc (19:1), and toluene-EtOAc-MeOH (30:8:1). Spots were visualized by spraying with 0.5 M H_2SO_4 and warming. Hplc was performed over LiChrosorp RP 18 (250 × 8 mm, 5 μM) with uv and ri detection using H_2O-MeOH (4:1) as an eluent. Gc-ms analyses were carried out on a Finnigan 9611 gc using a fused silica J&W, DB-5 column (30 × 0.25 mm, film 0.25 μm) coupled to a Finnigan 4515 ms. Ms were recorded on a Varian MAT 311 A in the ei mode; ir on a Perkin-Elmer 784 spectrometer using KBr discs for powders; optical rotation on a Perkin-Elmer 241 polarimeter. Nmr spectra were recorded on a Bruker AM 500 or a Bruker AM 250 instrument. Standard pulse sequences were used for COLOC and refocused INEPT. In order to optimize the polarization transfer from ^1H to ^{13}C during the COLOC experiment, the $^3J_{HC}$-value was determined by series of refocused INEPT spectra. In the case of compound **4**, optimal polarization transfer from the carbonyl carbons was observed for an apparent coupling constant of 6 Hz.

PLANT MATERIAL.—Roots of *T. villosa* [2n = 66 (= 6x)] were collected by the authors in July 1988, ca. 6 km south of Alter do Chao by road no. 245, Portugal. Plants were at the stage of fruit ripening. Voucher specimens (88-9) are deposited at the Department of Pharmacognosy, Royal Danish School of Pharmacy.

EXTRACTION AND ISOLATION.—Dried roots (428 g) were extracted with EtOH (96%) using an Ultra-Turrax to triturate the plant material. Concentration in vacuo of the extract yielded 52.2 g of a res-

idue, which was partitioned between H_2O and EtOAc (1:1). The organic phase was concentrated in vacuo to give 15 g, which was fractionated by cc using CH_2Cl_2/EtOAc mixtures of increasing polarities as eluents. Compounds **4** and **5** were further purified by cc using hexane/EtOAc mixtures of increasing polarities as eluents to give 400 mg of **4**, 206 mg of **5**, and 869 mg of a mixture of **4** and **5** in a ratio of 2:1. Compounds **2** and **3** were purified by cc using CH_2Cl_2-EtOAc (1:4) and hexane/EtOAc mixtures of increasing polarities as eluents to give 166 mg of **2**, 26 mg of **3**, and 43 mg of a mixture of **2** and **3** in a ratio of 1:1. The homogeneities of the compounds were verified by hplc.

CHARACTERIZATION OF **2**.—Colorless amorphous powder: $[\alpha]^{26}D$ −108° (MeOH, c = 0.29); ir 1790, 1735, 1640, 1230, 1130 cm^{-1}; ms *m/z* (rel. int.) $[M-HOAc]^+$ 446 (8), $[M-HOAc-CH_2CO]^+$ 404 (5), $[M-HOAc-CH_2CO-(Me)_2CCHCOOH]^+$ 304 (4), $[M-HOAc-CH_2CO-(Me)_2CCHCOOH-HOAc]^+$ 244 (22), $[M-HOAc-CH_2CO-(Me)_2CCHCOOH-HOAc-H_2O]^+$ 226 (33), $[(Me)_2CCHCO]^+$ 83 (100), 43 $[Ac]^+$ (50); ^1H nmr see Table 1; ^{13}C nmr see Table 2.

CHARACTERIZATION OF **3**.—Colorless amorphous powder: $[\alpha]^{26}D$ −113° (MeOH, c = 0.21); ir 1790, 1735, 1230 cm^{-1}; ms *m/z* (rel. int.) $[M-HOAc]^+$ 448 (15), $[M-HOAc-CH_2CO]^+$ 406 (25), $[M-HOAc-CH_2CO-C_2H_5(Me)CHCOOH]^+$ 304 (8), $[M-HOAc-CH_2CO-C_2H_5(Me)CHCOOH-HOAc]^+$ 244 (47), $[M-HOAc-CH_2CO-C_2H_5(Me)CHCOOH-HOAc-H_2O]^+$ 226 (91), $[C_2H_5(Me)CHCO]^+$ 85 (27), $[C_4H_9]^+$ 57 (87), $[Ac]^+$ 43 (100); ^1H nmr see Table 1; ^{13}C nmr see Table 2.

CHARACTERIZATION OF **4**.—Colorless amorphous powder: $[\alpha]^{26}D$ −129° (MeOH, c = 0.20); ir 3420, 1785, 1730, 1640, 1235, 1130 cm^{-1}; ms *m/z* (rel. int.) $[M-HOAc]^+$ 404 (2), $[M-HOAc-(Me)_2CCHCOOH]^+$ 304 (4), $[M-HOAc-(Me)_2CCHCOOH-HOAc]^+$ 244 (24), $[M-HOAc-(Me)_2CCHCOOH-HOAc-H_2O]^+$ 226 (6), $[(Me)_2CCHCO]^+$ 83 (100), $[Ac]^+$ 43 (38); ^1H nmr see Table 1; ^{13}C nmr see Table 2.

CHARACTERIZATION OF **5**.—Colorless amorphous powder: $[\alpha]^{26}D$ −50° (MeOH, c = 0.24); ir 3425, 1785, 1730, 1240 cm^{-1}; ms *m/z* (rel. int.) $[M-HOAc]^+$ 406 (13), $[M-HOAc-C_2H_5(Me)CHCOOH]^+$ 304 (10), $[M-HOAc-C_2H_5(Me)CHCOOH-HOAc]^+$ 244 (64), $[M-HOAc-C_2H_5(Me)CHCOOH-HOAc-H_2O]^+$ 226 (13), $[C_2H_5(Me)CHCO]^+$ 85 (48), $[C_4H_9]^+$ 57 (90), $[Ac]^+$ 43 (100); ^1H nmr see Table 1; ^{13}C nmr see Table 2.

METHANOLYSIS OF **2–6**.—A solution of the guaianolide in 14% BF_3 in MeOH was left overnight at room temperature. The reaction mixture was added to a saturated aqueous solution of NaCl and extracted with hexane. A gc-ms investigation revealed the presence of **9** and, in trace amounts, **8**. The hexane phase was concentrated in vacuo, and **9** was purified by cc using hexane/EtOAc mixtures of increasing polarities as eluents. The ^1H-nmr and ms data matched those reported for **9** (12).

ACETYLATION OF **4** AND **5**.—The compounds (10 mg) were dissolved in a solution of Ac_2O (13 μl) and 4-dimethylaminopyridine (50 mg) in CH_2Cl_2 (2.5 ml). The solution was left for 5 min at room temperature, and 4 M HCl (5 ml) was added. The organic phase was concentrated to give the acetylated derivatives of **4** and **5**, the ^1H-nmr spectra of which were superimposable on those of **2** and **3**, respectively.

IRRITANT TEST.—Each of the compounds **2–5** (10 μg in 10 μl of Me_2CO) was applied to the ears of mice. The extents of irritation were estimated 24 h later (18).

HISTAMINE RELEASE.—Peritoneal rat mast cells were incubated with the compounds **2–5** in concentrations up to 10 μM using the previously described protocol (1). The histamine assay described by Moldt *et al.* (19) was used.

ACKNOWLEDGMENTS

This work was supported by the Danish State Biotechnology Program 1987–1990. P.A. is grateful to the Italian C.N.R. for financial assistance. We thank Mrs. J. Grove Rasmussen for recording the 500 MHz nmr spectra with the instrument of the Danish Natural Sciences Research Council; Dr. J. Møller, University of Odense, for recording the eims; and Dr. F. Vester for recording the gcms. Part of the equipment for the histamine assay was a gift from the Carlsberg Foundation.

LITERATURE CITED

1. E. Norup, U.W. Smitt, and S.B. Christensen, *Planta Med.*, **52**, 251 (1986).
2. S.B. Christensen, E. Norup, U. Rasmussen, and J.Ø. Madsen, *Phytochemistry*, **23**, 1659 (1984).
3. T.G. Tutin, V.H. Heywood, N.A. Burges, D.M. Moore, D.H. Valentine, S.M. Walters, and D.A. Webb, "Flora Europaea," University Press, Cambridge, 1968, Vol. 2, p. 370.
4. U. Rasmussen, S.B. Christensen, and F. Sandberg, *Planta Med.*, **43**, 336 (1981).
5. E. Lemmich, B. Jensen, and U. Rasmussen, *Phytochemistry*, **23**, 809 (1984).

6. J. Pascual Teresa, J.R. Morán, J.M. Hernández, and M. Grande, *Phytochemistry*, **25**, 1167 (1986).
7. J. Pascual Teresa, J.R. Morán, A. Fernández, and M. Grande, *Phytochemistry*, **25**, 1171 (1986).
8. U.W. Smitt, C. Cornett, E. Norup, and S.B. Christensen, *Phytochemistry*, **29**, 873 (1990).
9. M. Holub, M. Budesínsky, Z. Smítalová, D. Saman, and U. Rychlewska, *Tetrahedron Lett.*, **25**, 3755 (1984).
10. Z. Smítalová, M. Budesínsky, D. Saman, and M. Holub, *Collect. Czech. Chem. Commun.*, **51**, 1323 (1986).
11. A. Lauridsen, C. Cornett, and S.B. Christensen, *Acta Chem. Scand.*, in press.
12. U.W. Smitt, P. Moldt, and S.B. Christensen, *Acta Chem. Scand., Ser. B*, **40**, 711 (1986).
13. J. March, "Advanced Organic Chemistry," 3rd ed., John Wiley & Sons, New York, 1985, p. 341.
14. Y. Matsubara, S. Takekuma, K. Yokoi, H. Yamamoto, and T. Nozoe, *Bull. Chem. Soc. Jpn.*, **60**, 1415 (1987).
15. G. Appendino and P. Gariboldi, *Phytochemistry*, **21**, 2555 (1982).
16. K. Volkác, Z. Samek, V. Herout, and F. Sorm, *Collect. Czech. Chem. Commun.*, **34**, 2288 (1969).
17. M. Holub and M. Budesínsky, *Phytochemistry*, **25**, 2015 (1986).
18. E. Hecker, in: "Methods in Cancer Research." Ed. by H. Busch, Academic Press, New York, 1971, Vol. 6, p. 439.
19. P Moldt, W.K. Andersen, and S.B. Christensen, *Agents Actions*, **24**, 35 (1988).

Received 6 *April 1990*